Contribution of chlorophyll fluorescence to the apparent vegetation reflectance

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ABSTRACT

Current strategies for monitoring the physiologic status of terrestrial vegetation rely on remote sensing reflectance data, which provide estimates of vigor based primarily on chlorophyll content. Chlorophyll fluorescence (ChlF) measurements offer a non-destructive alternative and a more direct approach for diagnosis of vegetation stress before a significant reduction in chlorophyll content has occurred. Thus, technology based on ChlF may allow more accurate carbon sequestration estimates and earlier stress detection than is possible when using reflectance data alone. However, the observed apparent vegetation reflectance (Ra) in reality includes contributions from both the reflected and fluoresced radiation. The aim of this study is to determine the relative contributions of reflectance and ChlF fractions to Ra in the red to near-infrared region (650–800 nm) of the spectrum. The practical objectives of the study are to: 1) evaluate the relationship between ChlF and reflectance at the foliar level for corn, soybean and maple; and 2) for corn, determine if the relationship established for healthy vegetation changes under nitrogen (N) deficiency.

To obtain generally applicable results, experimental measurements were conducted on unrelated crop and tree species (corn, soybean and maple) under controlled conditions and a gradient of inorganic N fertilization levels. Optical reflectance spectra and actively induced ChlF emissions were collected on the same foliar samples, in conjunction with measurements of photosynthetic function, pigment levels, and carbon (C) and N content. The spectral trends were examined for similarities. On average, 10–20% of Ra at 685 nm was actually due to ChlF. The spectral trends in steady state and maximum fluorescence varied significantly, with steady state fluorescence (especially red, 685 nm) showing higher ability for species and treatment separation. The relative contribution of ChlF to Ra varied significantly among species, with maple emitting much higher fluorescence amounts, as compared to corn and soybean. Steady state fluorescence from individual red and far-red emission bands (F685 and F740, respectively) and their ratio consistently enabled species separation. For corn, the relative ChlF fraction increased in concert with the nutrient stress levels from <2% for non-stressed foliage to >7% for severely N deficient plants. Steady state ChlF at 685 nm provided optimal N treatment separation. This study confirms the trends in the steady state red/far-red ratio (F685s/F740s) associated with N deficiency and vegetation stress, previously established using active single narrow band excitation.

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1. Introduction

An improvement in satellite-based assessments of the terrestrial vegetation's carbon (C) budget requires additional information on physiological condition, which can be obtained *directly* by measuring fluorescence emissions. Considering the rapid developments in fluorescence sensing capabilities (e.g., signal amplification and sensor miniaturization; laser induced technologies; and passive sensing technologies utilizing the Fraunhofer line depth approach), fluorescence has strong practical potential for monitoring vegetation status.

The red/far-red region (650–800 nm) of the apparent vegetative reflectance (Ra) spectrum typically includes the contribution of both reflectance and fluorescence (Lichtenthaler et al., 1986; Chappelle et al., 1999). A portion of the absorbed solar energy is utilized in the process of photosynthesis via biochemical reactions, while the absorbed energy not utilized in photosynthesis is either emitted as fluorescence at longer wavelengths or dissipated as heat. Vegetation fluorescence is emitted from the foliage throughout the ultraviolet to visible regions of the spectrum, with peaks occurring at 340, 445, 530, 685, and 740 nm (Lichtenthaler et al., 1986; Chappelle et al., 1999). While it has been assumed that fluorescence is a very small portion of Ra, further research is needed to quantify the contribution of fluorescence to Ra, especially in the red (685 nm) and far-red (740 nm) regions where the chlorophyll fluorescence (ChlF) emissions are maximal.

Commonly, laser induced fluorescence technologies apply a strong narrow pulsed excitation beam of illumination, and fluorescence emissions can be differentiated from reflectance through a fast gated detector (Corp et al., 1996, 2006). The magnitude of the emitted fluorescence strongly depends on the wavelength and intensity of the excitation energy. The photosynthetic pigments absorb radiation primarily in the 350–700 nm region, while their emittance occurs in broad peaks in the red (685 nm) and far-red (740 nm) regions of the spectrum. While active fluorescence techniques have established steady state ChlF as a reliable approach for diagnosis of vegetation physiological condition, very few studies have
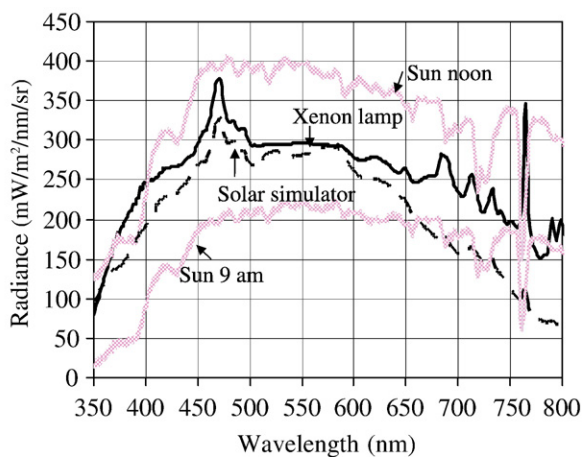
Fig. 1 – Solar spectra and radiation output of the Xenon lamp (300 W, 2001) and Solar simulator (300 W, 2004), used as reflectance illumination and ChlF excitation sources.
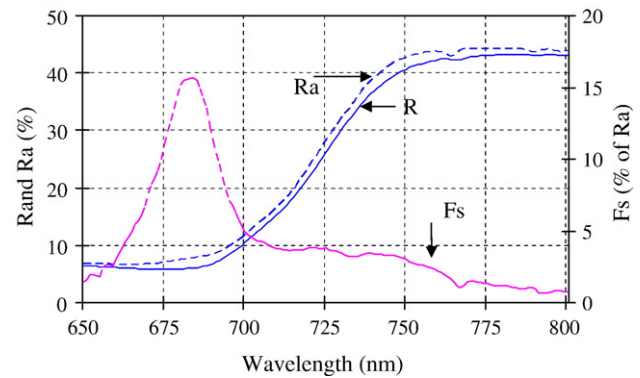
Fig. 2 – Vegetation reflectance (R, solid line), apparent reflectance (Ra, dashed line) and steady state fluorescence ($Fs_\lambda = Ra_\lambda - R_\lambda$, dotted line). Data from corn foliage (2001, ANOVA, LS Means).

validated the potential of solar induced ChlF (Theisen et al., 1994; Moya et al., 2004; Louis et al., 2005; Meroni and Colombo, 2006). Fluorescence derived using monochromatic excitation (e.g., 360 nm) in the red (F685) or far-red (F740), and their ratio (F685/F740), have successfully detected various types of vegetation stress, and enabled quantification of the amount

Table 1 – Differences in chlorophyll fluorescence parameters in maple, corn and soybean

Fluorescence parameter[†]	Species		
(F[‡])	Maple	Corn	Soybean
1. Fluorescence amount at 685 and 740 nm expressed as % of the incoming radiation			
F685max	4.58a	2.43b	1.82c
F685s	1.87a	0.95b	0.54c
F740max	8.34a	3.73b	3.70b
F740s	3.05a	1.99b	1.32c
F760max	8.57a	3.19	2.69b
F760s	3.46a	1.80b	1.12c
F685s/F740s	0.61a	0.48b	0.39c
F685s/F760s	0.54a	0.53a	0.48a
2. Fluorescence amount at 685 and 740 nm expressed as % of radiation reflected off the vegetation (Ra)			
F685max	44.80	30.12	23.48
F685s	21.87	10.06	8.72
F740max	11.22	8.13	6.94
F740s	5.20	2.86	2.06
F760max	7.51	5.96	5.13
F760s	3.61	1.84	1.04
3. Fluorescence amount at 685 and 740 nm ($mW\ m^{-2}\ sr^{-1}\ nm^{-1}$)			
F685max	9.87	4.99	3.32
F685s	3.42	1.49	1.44
F740max	11.34	7.62	7.52
F740s	5.36	2.47	2.46
F760max	8.29	6.19	6.00
F760s	3.89	1.62	1.33

[†] Means are compared within row, indicating significant differences among treatments with different letters (ANOVA).
[‡] F685max or F740max is the maximum fluorescence at 685 nm or 740 nm; F685s or F740s — steady state fluorescence at these bands.

of crop residue covering agricultural soil surfaces (Chappelle et al., 1999; Corp et al., 1996; McMurtrey et al., 1994; Middleton et al., 1996).

Using a full solar spectrum as an excitation source, the goals of this investigation are: 1) to determine the relative reflectance and ChlF fractions contributing to the cumulative vegetation radiance at 685 nm, 740 nm and 760 nm (i.e., contribution of ChlF to Ra) at the foliar level; and 2) to examine the variation in ChlF associated with species differences, or with nitrogen (N) deficiency in corn.

2. Methods

2.1. Vegetation material and treatments

Experimental measurements were acquired in 2001 and 2004 at the middle of the growing season at several field sites at the USDA Agricultural Research Center, Beltsville, MD. To simulate the varying rates of total seasonal atmospheric N deposition and provide a range in plant growth conditions, we used foliage from experimental plants under inorganic N treatments ranging from N deficiency to excess. Foliar samples were collected from corn (*Zea Mays* L., in 2001 and 2004), soybean (*Glycine max* (L.) Merr., in 2004) Illini cultivar and maple (*Acer rubrum* L., in 2004). Photosynthetic efficiency measurements were collected *in situ*. The uppermost fully expanded leaves or 3rd leaf from terminal were excised from the plant canopy, immediately placed in water filled sample holders, and transported to the laboratory for spectral measurements, pigments, inorganic C and N determinations (Campbell et al., 2007).

Corn samples were collected from an intensive test site for a multi-disciplinary project entitled "Optimizing Production Inputs for Economic and Environmental Enhancement" (OPE)

to develop new farming strategies. The experimental design was a randomized complete block with treatment groups of 280 (150% of optimal), 140 (optimal, 100%), 70 (50%), and 0 (0%) kg N/ha. Measurements were acquired in August at the R3 grain fill reproductive stage (18–22 days after pollination, when N deficiency can cause light weight grain or severe stress can abort the kernels; Ritchie et al., 1993).

The soybean was grown in a sterile perlite growth medium where no rhizobium species were allowed to infect the roots. Nutrient solutions from 0.002 M (N deficiency) to 0.005 M (optimal plant growth) were applied weekly for 45 days. These levels correspond to 100%, 75%, 50%, 25%, and 0% of the N required for optimal growth. The plants were measured after eight weeks of growth.

Maple foliar samples were collected from a multiyear experiment where six-year old saplings were planted in the ground in 2001 and treated with N in the form of urea, with varying concentrations from 0.004 M to 0.001 M.

2.2. Spectral measurements and data analysis

To determine the amount of fluorescence included in Ra, spectral measurements were conducted using procedures established by Kim et al. (1993) and further developed by Zarco-Tejada (2000). High spectral resolution measurements were acquired using an ASD-FR FieldSpec® Pro, with 3 nm resolution at full width half maximum and 1.4 nm sampling interval in the 350–800 nm region (Analytical Spectral Devices, Inc.; Boulder, CO). The sensor's foreoptic view angle was 8° and the view area was approximately 2 cm². A spectralon panel was used as a reference standard. A 300 W xenon arc lamp and a set of neutral density filters (in 2001) and a 300 W solar simulator (Oriel 91160A; Newport Stratford Inc., Stratford, CT) outfitted with a Global Air Mass Filter (Oriel 81080; Newport Stratford Inc., Stratford, CT), were used to simulate constant solar radiation,
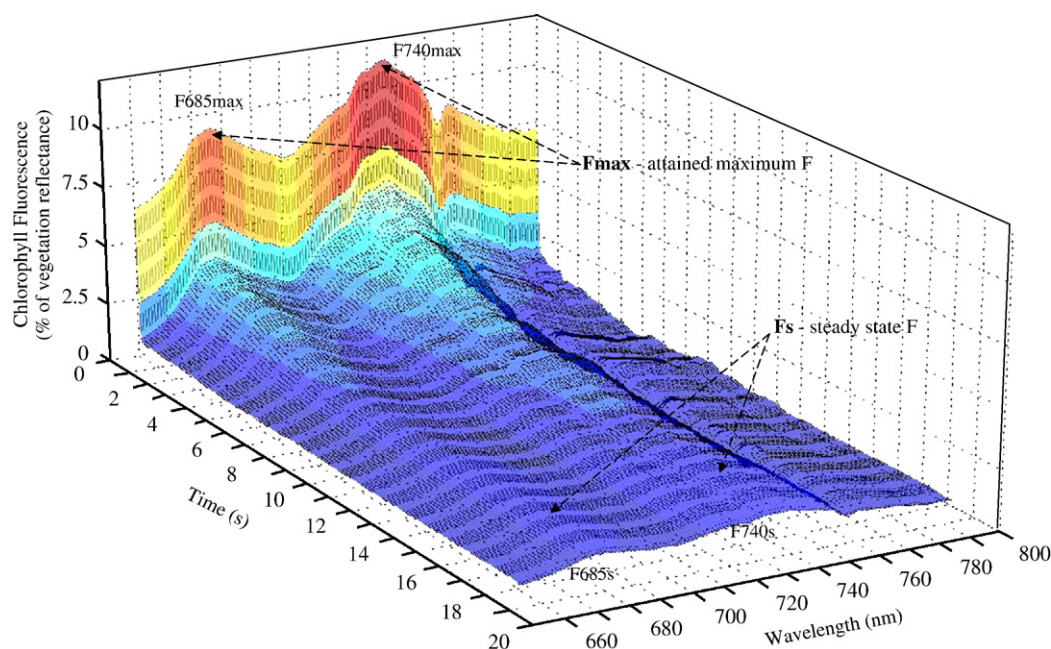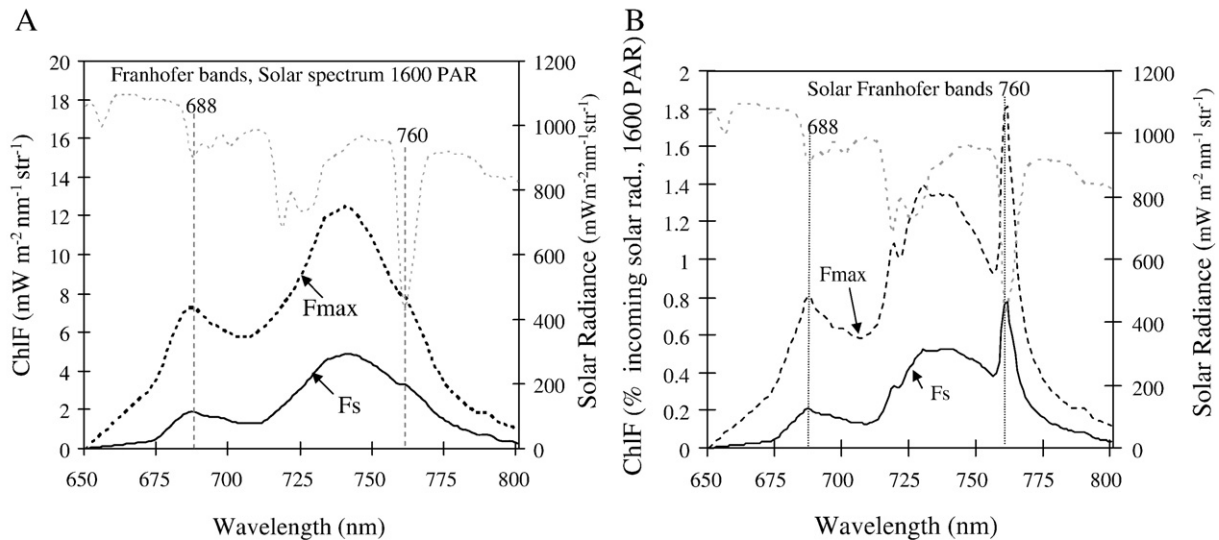

Fig. 3 – Time resolved ChlF spectra (% of R) of a corn foliage is shown (*Zea Mays* L.). The spectra were acquired after 5 min of dark adaptation of the sample at 0.01 seconds interval.

Fig. 4 – Maximum and steady state chlorophyll fluorescence (Fmax and Fs), shown for corn foliage (*Zea Mays* L., ANOVA, LS Means, 2001 and 2004 data). The spectra were acquired at 0.01 second intervals after 5 min dark adaptation of the samples. Fluorescence levels decreased significantly from Fmax (dashed line) to Fs (solid line) in 15–20 s, after which they remained relatively constant. Chlorophyll fluorescence emissions are shown: A. in energy units (mW m^{-2} nm^{-1} sr^{-1}), where ChlF at 688 nm declined from approximately 7.25 (Fmax) to 2.10 (Fs); and B. as a percent of incoming PAR (1600 µmol m^{-2} s^{-1}), where the solar Fraunhofer features at 688 nm and 760 nm offer a potential for passive ChlF measurements.

resembling the spectrum in the Washington, DC area in mid July (Fig. 1). A Schott RG 665 long pass filter was used to prevent fluorescence induction from below 665 nm. The illumination setup was used in one of two modes: with the RG 665 filter blocking the light source to measure reflectance; and without the filter to measure Ra (reflectance + ChlF, Fig. 2). Samples were dark-adapted for 5 min immediately before initiating the Ra measurements. Recording data every 0.01 s for 25 s, spectra were obtained across the 650–800 nm region.

Fluorescence in the 650–800 nm region was calculated as the difference between the measurements acquired with the long pass filter to measure reflectance only (R) and without the filter to measure Ra (Fig. 2), as:

$$F_\lambda = Ra_\lambda - R_\lambda.$$

The maximal ChlF (Fmax) and steady state ChlF (Fs) were examined to compare values obtained among species or N treatment levels (corn only) at 685 nm, 740 nm and 760 nm. Statistical analysis was performed using general linear model analysis of variances (GLM, ANOVA, SYSTAT 8.0; SPSS Inc., 1997) and the significance of the differences was determined by the Tukey–Kramer test. Estimates of Fmax and Fs for ChlF were expressed as: (i) a percentage of the incoming radiation; (ii) as a percentage of the radiation reflected from the vegetation; and (iii) in energy units (mW m^{-2} nm^{-1} sr^{-1}) in Table 1.

3.   Results and discussion

In the red-edge region (680–725 nm) vegetation has relatively low reflectance (Fig. 2), due to strong chlorophyll *a* absorption. In all cases, the radiation measured as Ra without the long pass filter was consistently higher than when measured with

the filter, which blocked ChlF emissions (Figs. 2, 4 and 5). Although the absolute ChlF was relatively small, both maximum and steady state ChlF (Fmax and Fs) contributed 5–15% to Ra (Fig. 2). The time required to attain Fs from Fmax (Figs. 3 and 4) varied between 15 to 20 s, but was not found to be significantly different among either species or N treatment.

3.1.   Variation in ChlF with species type

The relative contribution of Fmax and Fs to Ra at the peak wavelengths varied significantly among species, with maple leaves exhibiting significantly higher emissions than both
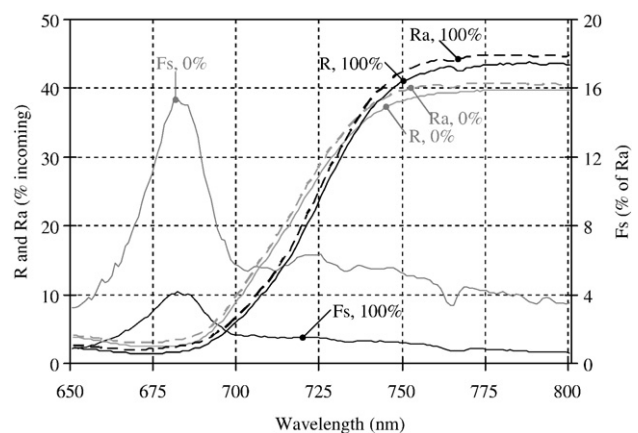
Fig. 5 – While the contribution of steady state fluorescence (Fs) to the apparent vegetation reflectance (Ra = R + Fs) may not appear significant, it could account for 4–15% of Ra in the 670–690 nm region. Reflectance (R) and Ra (dashed lines) for healthy (N = 100%, black lines) and nitrogen deficient (N = 0%, grey lines) corn foliage (ANOVA, LS Means, 2001 data).

Table 2 – Changes in chlorophyll fluorescence parameters for corn associated with N treatment (2001 and 2004 data)					
Fluorescence parameter [†]	Nitrogen treatment levels [§]				
(F [‡])	0	50	100	150	r^2
1. Fluorescence at 685 and 740 nm expressed as % of the incoming radiation					
F685max (2001)	2.48ns	2.15ns	2.01ns	2.15ns	0.55
F685max (2004)	3.42b	3.41b	3.58b	4.45a	0.72
F685s (2001)	0.87a	0.59b	0.21c	0.52b	0.74
F685s (2004)	1.48b	2.03a	0.65c	1.76b	0.72
F740max (2001)	2.28c	2.88ab	2.71b	3.20a	0.61
F740max (2004)	5.71c	6.24a	6.51a	11.53a	0.69
F740s (2001)	0.82a	0.84a	0.58b	0.83a	0.62
F740s (2004)	3.44b	4.5a	2.55c	3.45b	0.75
2. Fluorescence at 685 and 740 nm expressed as % of radiation reflected off the vegetation (Ra)					
F685max (2001)	29.81	24.83	35.70	38.90	
F685max (2004)	33.87	32.05	40.68	44.87	
F685s (2001)	15.20	8.38	5.54	4.26	
F685s (2004)	17.89	19.76	13.26	9.16	
F740max (2001)	8.20	7.14	9.28	10.49	
F740max (2004)	8.76	9.49	10.66	14.11	
F740s (2001)	4.97	4.01	2.49	3.02	
F740s (2004)	6.29	5.99	6.62	7.07	
3. F at 685 and 740 nm ($mW\ m^{-2}\ sr^{-1}\ nm^{-1}$)					
F685max (2001)	4.60	3.82	5.01	4.81	
F685max (2004)	6.21	5.87	7.46	8.22	
F685s (2001)	1.82	1.31	1.12	1.25	
F685s (2004)	2.98	3.29a	2.68	2.69	
F740max (2001)	6.21	7.13	8.07	9.12	
F740max (2004)	7.62	8.25	9.27	12.27	
F740s (2001)	3.43	3.49	2.16	3.26	
F740s (2004)	5.47	5.21	5.76	6.15	

[†] Means are compared within a row, indicating significant differences among treatments with different letters (ANOVA).
[‡] F685max or F740max is the maximum fluorescence at 685 nm or 740 nm; F685s or F740s — steady state fluorescence at these bands.
[§] Nitrogen treatment levels (% of optimum, LS Means).

sensitivity sharply decreases as the far-red measurement is collected away from the ChlF peak at 740 nm (Fig. 4 A), as in the case of the 760 nm telluric O_2 feature used for the remote sensing retrieval of solar induced ChlF.

The shape of the spectra in relative units (% incoming PAR) differs significantly from the spectra expressed in absolute energy units (Fig. 4). Some of these variations, as commonly expressed for reflectance as percentage of the incoming radiation (Fig. 4 B), could be associated with the spectral characteristics of the excitation source.

3.2. Variation in ChlF with N treatment in corn

Based on both 2001 and 2004 data, 10–25% at 685 nm and 2–6% at 740 nm of Ra was due to steady state ChlF (Fs, Fig. 5 and Table 2). These results comply with the range in solar induced ChlF at the same bands reported by Corp et al. (2006) for corn under N treatments, using a different measurement approach. The amount of steady state ChlF varied in association with the N treatments, and significantly affected the relationship between ChlF and reflectance (Fig. 5).
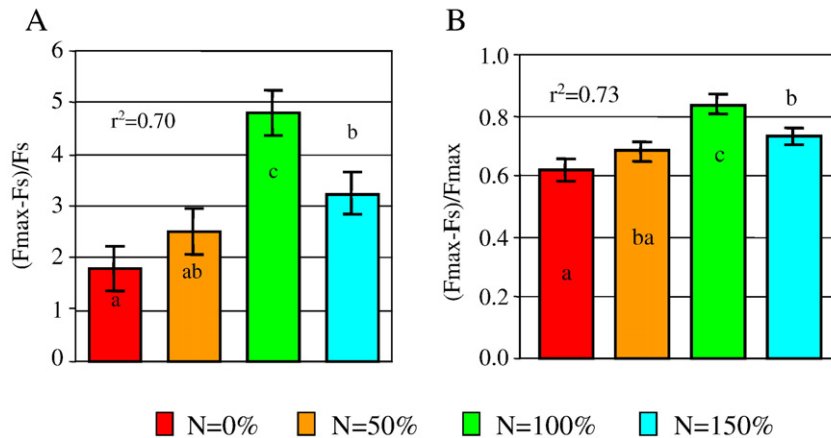
A slight decrease in Fmax was observed in association with N deficiency (more pronounced in the 2004 measurements), while the relative Fs fraction of the Ra increased in concert with the N stress levels (Table 2). Optimal separation of the N treatments in corn foliage was achieved by F685s (Table 2). Overall, the variation in corn ChlF intensity associated with the N treatments was lower than the variation observed among species.

Using a single excitation band, the red/far-red (F685s/F740s) ChlF ratio has been shown to increase in association with vegetation stress, as chlorophyll content decreases or as photosynthetic rate declines (Chappelle et al., 1999; Lichtenthaler and Rinderle, 1988; McMurtrey et al., 1994; Middleton et al., 2005). The pattern observed in this ratio using our data, which were acquired under a full solar excitation spectrum (Fig. 6), complied well with the trends suggested in the literature and associated with N deficiency or excess N supply.

Variable ChlF (Fv) is a well established induction kinetic parameter calculated as the difference between Fs and Fmax.
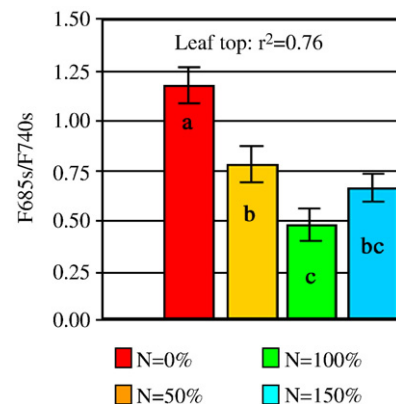
Fig. 6 – Differences in the steady state fluorescence (Fs) red/far-red (685/740 nm) ratio were associated with N treatments (ANOVA, LS Means and Std. Errors, corn data 2001 and 2004). ChlF induced with full solar spectrum was indicative of N availability in corn.

corn and soybean (Table 1). At 685 nm the ChlF contribution for Fs was 8.7–44.8% of Ra; at 740 nm it amounted to 2.8–11.2%; and at 760 nm was 1–7.5% (Table 1). These values agree with those reported for a variety of species under ambient solar illumination conditions (Corp et al., 2006; Dobrowski et al., 2005; Louis et al., 2005; Meroni and Colombo, 2006; Theisen, 2000). Fs and its ratios (F685s/F740s; F685s/F760s) consistently allowed for species separation whereas Fmax did not (Table 1).

The discrimination among species using a full solar spectrum to obtain the steady state red/far-red ChlF ratio (F685s/F740s nm, Table 1) agrees with results from earlier studies using excitation by a single blue (460 nm), green (530 nm) or red (650 nm) band (Chappelle et al., 1999; Corp et al., 1996; Middleton et al., 1996). When using the F685s/F760s ratio, because F760s is located at the shoulder of the spectral curve (Fig. 2), the fluorescence intensity and the sensitivity of the red/far-red ratio were much lower and species separation was not achieved (Table 1). While the red/far-red ratio obtained using simulated solar excitation can provide similar results to the monochromatically excited fluorescence, its

Fig. 7 – Variable ChlF (Fv) is presented for F685, where Fv is expressed relative to Fs [F685v(s)=(Fmax – Fs)/Fs] or relative to Fmax [F685v(max)=(Fmax – Fs)/Fmax]. The optimal (100% N) treatment produced significantly higher values than other treatments: A] F685v(s), where r^2=0.70; and B] F685v(max), where r^2=0.73. N deficiency is evident for F685v(s) in [A], where values are ≤2.9 for the two lower N groups.

Fv can be expressed as a function of Fs [Fv(s)=(Fmax – Fs)/Fs] or as a function of Fmax [Fv(max)=(Fmax – Fs)/Fmax] at F685 and F740. Significant differences in these ratios were obtained for F685 (e.g., F685v(s) and F685v(max)), as shown in Fig. 7. The highest Fv(s) and Fv(max) for F685 were observed from the optimal corn treatments (N=100%, Fig. 7). The trends in Fv(s) and Fv(max) (Fig. 7) for F685 comply with reports of previous studies (Lichtenthaler et al., 1986; Mohammed et al., 1995) and illustrate the utility of the current measurement approach for achieving both steady state ChlF measurements and induction kinetics parameters. Both Fv(s) and Fv(max) (Fig. 7) have been suggested as indicators of photosynthetic efficiency and function (Lichtenthaler and Rinderle, 1988; Lichtenthaler et al., 1986). We found that Fv(s) values <2.5 were associated with N deficiency (0% and 50%, Fig. 7), which could serve as an indicator of inefficiency of CO_2 assimilation associated with N deficiency (Mohammed et al., 1995).

4. Conclusions

Understanding the dynamic processes of photosynthesis, C sequestration and vegetation growth require methods that quantify the instantaneous changes in response to alteration in the local environmental conditions. Dynamic methods are needed if the full potential of ChlF analysis is to be realized. Direct measurement of ChlF at a distance has been an objective of many remote sensing investigations (both passive and laser-based active methods) and has proven challenging since the relatively weak ChlF emissions from the vegetation must be differentiated from more intense reflectance signals. Using the dark lines in the solar spectrum reaching the Earth (reported by Joseph Fraunhofer in 1817) to differentiate fluorescence from the solar continuum by interferometer-type passive satellite systems offers a potential for continuous orbital observation. While this technology and methods are routinely applied for atmospheric composition analysis and determinations, the need for rigorous validation of the scientific merit of these data have rated a

number of research efforts risky and have prevented their support.

This investigation confirms for three species the significant contribution of ChlF to the apparent reflectance (Ra) in the red and far-red (650–800 nm) regions. On average, 10–25% of Ra at 685 nm and 2–6% at 740 nm was actually due to steady state fluorescence. At 685 nm, the maximum fluorescence achieved was ∼2–4% and the steady state was 0.5–1.5% of the incoming PAR. At 740 nm the fluorescence values were higher with maximum fluorescence at ∼4–6% and steady state at ∼1–2% of incoming PAR. The relative steady state fluorescence fraction at 685 nm increased in concert with the nutrient deficiency levels in corn leaves. The relationship between foliar fluorescence and reflectance was significantly affected by the physiological status of the vegetation, for corn under N treatments.

Using a simulated solar excitation spectrum, this study confirms the trends in the red/far-red (F685s/F740s), Fv(s) and Fv(m) ratios associated with vegetation stress as previously established. Species and/or N treatment levels were discriminated with all three of the ChlF kinetic parameters studied: Fs, Fmax and Fv. ChlF parameters associated with F685 proved more sensitive than those for F740 or F760. The steady state ratio for Fv(s) was especially useful for discerning plants grown in low N. These findings suggest that passively determined ChlF can monitor the physiological status of vegetation and provide results comparable to the large body of data available from actively induced fluorescence research. Future research using solar excitation is needed to validate for a variety of species, environmental and solar conditions the established (using single excitation band) trends and relationships of ChlF to photosynthetic function and vegetation stress.

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